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Award Number: W81XWH-04-1-0491

TITLE: Mechanisms of Chemoresistance in Breast Cancer Cells

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REPORT DATE: May 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2005	Annual (1 May 2004 - 30 Apr 2005)

4. TITLE AND SUBTITLE
Mechanisms of Chemoresistance in Breast Cancer Cells

5. FUNDING NUMBERS
W81XWH-04-1-0491

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Our previous studies showed that both glucosylceramide synthase (GCS) and P-glycoprotein (P-gp) are overexpressed in Adriamycin-resistant human breast cancer cells, MCF-7-AdrR cells. When these cells were transfected with GCS antisense (asGCS), a stable 30% decrease in GCS activity was obtained. Experiments with paclitaxel (Taxol) showed that intracellular levels of drug were 8.6-fold greater in the asGCS-transfected cell line, MCF-7-AdrR/asGCS, compared to MCF-7-AdrR cells. In assessing P-gp, we observed a dramatic decrease in the level of MDR1 expression (80%) by RT-PCR that translated into a similar decrease in P-gp protein levels. To confirm the influence of GCS on MDR1 expression, we inhibited GCS. Treatment of MCF-7-AdrR cells with GCS inhibitor, 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), or with GCS siRNA, produced a significant decrease in MDR1 mRNA levels compared to untreated. These results were used in manuscript for publication, which showed that P-gp expression can be downregulated by either GCS antisense transfection or chemical inhibition of GCS. In order to determine whether overexpression of GCS is a general characteristic of chemotherapy resistance, we assessed GCS expression and glycolipid levels in Adriamycin-, cisplatin-, etoposide-, and paclitaxel- resistant breast cancer cells.

14. SUBJECT TERMS
Clucosylceramide synthase, P-glycoprotein, chemotherapy

15. NUMBER OF PAGES
21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Poor response to chemotherapy is a major clinical problem, and in most instances drug resistance is underlying cause (1). This is a most undesirable situation, and patients and oncologists would welcome its possible correction. In breast cancer nearly 50% of patients demonstrate primary and/or secondary resistance to Adriamycin (2). Several mechanisms of drug resistance are being examined, and avenues to reverse resistance are being sought. Research strategies in this area have become increasingly aimed at molecular targets such as P-glycoprotein (P-gp), multidrug resistance associated protein (MRP), topoisomerase, and Bcl-2 protooncogene, to name a few. Overexpression of the membrane efflux transporter, P-gp is one of the most consistent biological alterations in drug resistance (1,3). P-gp (170 kDa) is the product of the *mdr1* gene, an energy-dependent pump that reduces the intracellular concentration of specific anticancer drugs, and it has been studied extensively. Our approach to drug resistance is new and involves ceramide metabolism. The area has been reviewed (4,5). Several front-line anticancer agents elicit the formation of ceramide, a proapoptotic lipid messenger (6,7), by activating either the *de novo* or sphingomyelinase pathways of ceramide production (4,7). Drugs that induce cellular ceramide generation include anthracyclines (Adriamycin), *Vinca* alkaloids, etoposide (VP-16), paclitaxel, and fenretinide (4-HPR). If ceramide formation in response to drug treatment is blocked, then the cytotoxic impact of the drug is largely reduced (8,9). This demonstrates ceramide's role in drug responses. Ceramide added directly to cells circumvents the enzyme route of ceramide formation and promotes an apoptotic cascade directly (9,10). Our group showed that increased cellular capacity for ceramide glycosylation, catalyzed by glucosylceramide synthase (GCS), is associated with chemotherapy resistance in cancer cells (11-15). In cultured breast cancer cells, sensitivity to anthracyclines and taxanes can be decreased or increased simply by manipulation of GCS activity (13,16,17). For example, transfection of drug-sensitive MCF-7 breast cancer cells with GCS cDNA confers resistance to Adriamycin (16), and tranfection of multidrug-resistant MCF-7-AdrR breast cancer cells with antisense GCS (asGCS) increases cell sensitivity to chemotherapy by a factor of 28-fold for Adriamycin, more than 100-fold for vinblastine, and more than 200-fold for paclitaxel (13).

BODY

Training

-Real-Time RT-PCR Analysis

During the past year, I have learned the technique of real-time RT-PCR. I have worked to determine the best conditions to quantify gene expression in our system. I have designed sequences of primers and probes that we actually use, and I have also compared different reagents. Using this technique, I have accurately measured the levels of GCS and MDR1 expression in various cancer cell lines, under different experimental conditions.

-Deviations from the original statement of work

The aim of the study is to understand the role of glucosylceramide synthase (GCS) in the mechanism of drug resistance in breast cancer. We used a slightly different approach than we planned when we wrote the project, namely, we incorporated the use of another cell line, MCF-7-AdrR/asGCS. This cell line is an Adriamycin-resistant breast cancer cell line stably transfected with GCS antisense. In this cell line, GCS activity is knocked down by 30%. This tool helped us to understand the implications of GCS in the mechanisms of drug resistance in breast cancer cells, in particular as regards P-gp. This research allowed us to make important discoveries on the connection between GCS and P-gp, and this work has been reported in a publication (18). Moreover, this work can be related to task three of the statement of work: "Determine whether overexpression of GCS and overexpression of P-gp are allied or dissociated".

Key Research Accomplishments

1. **Influence of asGCS transfection on paclitaxel-induced ceramide accumulation.**
   Using mass analysis, we found that ceramide production in response to paclitaxel (1.0 μmol/L) was enhanced 3-fold in MCF-7-AdrR/asGCS cells compared with parental MCF-7-AdrR cells after 24 hour treatment. This shows that ceramide glycosylation is retarded by antisense GCS (because of high free ceramide levels).

2. **Chemotherapy uptake in MCF-7-AdrR and MCF-7-AdrR/asGCS cells.**
   Experiments with radiolabeled chemotherapy drugs showed that after 60 minutes, intracellular levels of paclitaxel were 8.6-fold greater in MCF-7-AdrR/asGCS cells compared with MCF-7-AdrR cells. This means that either drug uptake is enhanced or efflux is subdued.

3. **P-gp expression in MCF-7-AdrR and MCF-7-AdrR/asGCS cells.**
   Because of pronounced differences in drug levels in the two cell lines, we assessed P-gp expression by mRNA and protein determinations. The level of MDR1 mRNA, evaluated by reverse RT-PCR was dramatically lower in MCF-7-AdrR/asGCS cells compared with MCF-7-AdrR cells. We confirmed this by Western blot. Whereas, MCF-7-AdrR cells contained characteristically elevated levels of P-gp, MCF-7-AdrR/asGCS cells displayed a dramatic decrease (~80%) in P-gp levels.

4. **Influence of PPMP on MDR1 gene expression in MCF-7-AdrR cells.**
   To determine whether decreased MDR1 expression in asGCS cells was really due to GCS inhibition and/or downregulation, we evaluated the influence of D-L-*threo*-PPMP, a chemical inhibitor of GCS, on MDR1 expression in MDR1-rich MCF-7-AdrR cells. PPMP greatly diminished the expression of MDR1 in MCF-7-AdrR cells, with demonstrated stereospecificity. Unlike D-L-*threo*-PPMP, D-L-*erythro*-PPMP is not a GCS inhibitor, and this stereoisomer had no influence on MDR1 expression.

   Real-time RT-PCR showed that MDR1 expression in MCF-7-AdrR cells treated with D-L-*threo*-PPMP and D-L-*erythro*-PPMP was reduced by 58% and 12%, respectively, compared with untreated MCF-7-AdrR cells.

5. **Influence of GCS siRNA on MDR1 gene expression in MCF-7-AdrR cells.**
   To reinforce the results obtained with PPMP and to confirm that changes in MCF-7-AdrR/asGCS cellular MDR1 expression were not due to clonal artifacts, we used GCS siRNA to treat MCF-7-AdrR cells. siRNA was introduced into cells using lipofectAMINE in serum-free medium for 4 hours. After 48 hours, both GCS and MDR1 mRNA were dramatically decreased by GCS siRNA compared with lipofectAMINE only controls. The siRNA had no effect on expression levels of α-actin.

6. **GCS expression in chemotherapy-resistant breast cancer cells.**
For this study, we used several breast cancer cell lines:
- Adriamycin-resistant cells, MCF-7-AdrR; paclitaxel-resistant cells MCF-7/PtxR30, and the matched wild type MCF-7.
- cisplatin-resistant cells, MCF-7/CDDP, and the matched wild-type, MCF-7/P.
- etoposide-resistant cells, MCF-7/VP, and the matched wild-type, MCF-7/F.

The levels of GCS mRNA were surveyed by RT-PCR. The highest GCS expression was observed in MCF-7-AdrR cells compared to wild-type breast cancer cells, whereas aGCS expression in MCF-7/CDDP cells was only slightly higher compared to MCF-7/P. A decrease in GCS mRNA was observed in MCF-7/VP cells compared to MCF-7/F cells.

To better quantitate GCS expression, we employed realtime RT-PCR.

GCS mRNA was 2.2- and 1.6-fold higher in MCF-7-AdrR and MCF-7/CDDP cells, respectively, compared to their wild-types. GCS expression in etoposide resistant cells, MCF-7/VP, was lowered by 36% compared to MCF-7/F.

7. **Determination of glucosylceramide (GC) mass in drug-resistant breast cancer cells.**
For GC quantitation, total lipids were extracted and analyzed by thin-layer chromatography (TLC) using a solvent system containing chloroform/methanol/NH_4OH (70:20:4, v/v/v). Approximately 250 µg of lipid were loaded per lane. After development, TLC plates were sprayed with sulfuric acid and heated (30 min, 180°C). The GC chars were quantitated by scanning densitometry, using a GC standard curve (0.5-5.0 µg). The results showed that GC mass was 3-fold higher in MCF-7-AdrR cells compared to the wild-type counterpart (3.95 versus 1.25 µg GC), and 1.76-fold higher in MCF-7/CDDP compared to wild-type counterpart (0.75 versus 0.425 µg). We found that GC mass decreased by 40 and 84% respectively, in etoposide- and paclitaxel-resistant cells, compared to the wild-type cells.

Key Research Accomplishments
- Mastered technique of real-time RT-PCR.
- Determined that paclitaxel induces ceramide accumulation in MCF-7-AdrR/asGCS cells.
- Determined that chemotherapy uptake is much higher in MCF-7-AdrR/asGCS cells compared to MCF-7-AdrR cells.
- Demonstrated that P-gp expression is lower in MCF-7-AdrR/asGCS cells compared to MCF-7-AdrR cells.
- Determined that PPMP diminishes the MDR1 expression in MCF-7-AdrR cells.

- Demonstrated that GCS siRNA decreases MDR1 gene expression in MCF-7-AdrR cells.
- Determined that GCS is overexpressed in Adriamycin- and cisplatin-resistant breast cancer cells, but not in etoposide-resistant cells, compared to wild type.
- Determined that GC mass is elevated in Adriamycin-, and in cisplatin-resistant cells, compared to wild-type cells, but it is diminished in etoposide- and paclitaxel-resistant cells compared, to wild-type cells.

Reportable Outcome

Oral Presentation
"Glucosylceramide synthase blockade downregulates P-glycoprotein and resensitizes multidrug-resistant breast cancer cells to anticancer drugs." Third International Charleston Ceramide Conference. Charleston, SC, March 2-6, 2005.

Publication
"Glucosylceramide synthase blockade downregulates P-glycoprotein and resensitizes multidrug-resistant breast cancer cells to anticancer drugs." Cancer Res., 2005, 65:3861-7.

CONCLUSION

In conclusion, our work shows that limiting GCS activity by either GCS antisense transfection, or PPMP treatment down-regulates the expression of P-gp. Moreover, in certain breast cancer cells resistant to chemotherapy (13), we found that overexpression of GCS correlated with an elevation of GC mass. This suggests that lipids play a role in multidrug resistance and that targeting glycolipid biosynthesis could be a promising approach for enhancing chemotherapy.

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APPENDICES

Glucosylceramide Synthase Blockade Down-Regulates P-Glycoprotein and Resensitizes Multidrug-Resistant Breast Cancer Cells to Anticancer Drugs

Valérie Gouazé, Yong-Yu Liu, Carlton S. Prickett, Jing Y. Yu, Armando E. Giuliano, and Myles C. Cabot

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Abstract

Overexpression of glucosylceramide synthase (GCS), a pivotal enzyme in glycolipid biosynthesis, contributes to cancer cell resistance to chemotherapy. We previously showed that transfection of doxorubicin-resistant MCF-7-AdrR cells with GCS antisense restored cell sensitivity to doxorubicin and greatly enhanced sensitivity to vinblastine and paclitaxel. In that study, doxorubicin promoted generation of ceramide in MCF-7-AdrR/GCS antisense cells; the present study implicates factors in addition to ceramide that augment sensitivity to chemotherapy. Although GCS antisense cells showed enhanced ceramide formation compared with MCF-7-AdrR when challenged with paclitaxel, GCS antisense cells also showed a 10-fold increase in levels of intracellular drug (paclitaxel and vinblastine). In addition, transfected cells had dramatically decreased expression (80%) of P-glycoprotein and a 4-fold decrease in the level of cellular gangliosides. Chemical inhibition of GCS produced the same effects as antisense transfection: exposure of MCF-7-AdrR cells to the GCS inhibitor 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP, 5.0 μmol/L, 4 days) decreased ganglioside levels, restored sensitivity to vinblastine, enhanced vinblastine uptake 3-fold, and diminished expression of MDR1 by 58%, compared with untreated controls. A similar effect was shown in vinblastin-resistant KB-V0.01 cells; after 7 days with PPMP (10 μmol/L), MDR1 expression fell by 84% and P-glycoprotein protein levels decreased by 50%. MCF-7-AdrR cells treated with small interfering RNAs to specifically block GCS also showed a dramatic decrease in MDR1 expression. This work shows that limiting GCS activity down-regulates the expression of MDR1, a phenomenon that may drive the chemosensitization associated with blocking ceramide metabolism. The data suggest that lipids play a role in the expression of multidrug resistance. (Cancer Res 2005; 65(9): 3861-7)

Introduction

Development of resistance to chemotherapeutic agents is a major concern in cancer patients. Resistance to chemotherapy is associated with myriad mechanisms that decrease drug cytotoxicity. Two members of the large family of ABC transporters confer multidrug resistance (MDR) in human cancer cells: P-glycoprotein and multidrug resistance protein (MRP). P-glycoprotein, a membrane-resident glycoprotein encoded by the *MDR1* gene, decreases the intracellular concentration of anticancer agents by acting as a drug efflux pump (1, 2). P-glycoprotein exports many types of chemotherapy drugs, including *Vinca* alkaloids, anthracyclines, paclitaxel, actinomycin D, and epipodophyllotoxins. Like P-glycoprotein, MRP is a transport protein (3); however, the transport of unconjugated chemotherapeutic agents by MRP seems to require glutathione. MDR in tumors can also be caused by overexpression of proteins such as antiapoptotic proteins belonging to the Bcl-2 family (4, 5) and by loss of tumor suppressor protein p53 (6, 7). Others factors responsible for chemotherapy resistance include reduction of topoisomerase II activity (8), modification of glutathione *S*-transferase activity (9), and up-regulation of rafts and caveolae, which are glycosphingolipid-enriched constituents of microdomains (10).

Glucosylceramide synthase (GCS) catalyzes the first glycosylation step in the biosynthesis of glycosphingolipids (11, 12). This central enzyme of ceramide metabolism has also been implicated in MDR (13). Glycosphingolipids, including glucosylceramide, lactosylceramide, and gangliosides, play an essential role in cell development, cell death, tumor progression, and pathogen/host interaction (13, 14). In addition, membrane gangliosides can decrease the sensitivity of human melanoma cells to ionizing radiation (15). In that study, radioresistant melanoma cells were made radiosensitive by exposure to either fumonisin B_1, which blocks ganglioside biosynthesis at the juncture of ceramide synthase, or *Vibrio* cholerae neuraminidase, which cleaves cell surface gangliosides. Conversely, adding bovine brain GM1 to radiosensitive melanoma cells conferred radioresistance (15). Targeting glycolipid metabolism has proven useful in altering chemotherapy responses in numerous human tumor cell lines (13, 16–18).

In previous studies, we increased the level of MDR by transfecting doxorubicin-resistant human breast cancer cells (MCF-7-AdrR) with GCS, and we enhanced cellular sensitivity to anthracyclines, *Vinca* alkaloids, and taxanes by transfecting MCF-7-AdrR cells with GCS antisense (16, 19). Although doxorubicin treatment of GCS antisense transfectants increased intracellular levels of ceramide (16), which is a second messenger of apoptosis, the extremely high sensitivity of MCF-7-AdrR/GCS antisense cells to *Vinca* alkaloids and taxanes suggested the participation of mechanisms other than ceramide signaling in cellular responses. We have observed equivalent intracellular levels of rhodamine-123 in MCF-7-AdrR/GCS antisense cells and in rhodamine-123–exposed MCF-7 parental cells, which indicates that GCS antisense transfection reverts drug retention in MCF-7-AdrR cells on a par with the drug-sensitive phenotype (16, 19). Because rhodamine-123 is a substrate for P-glycoprotein, we began to investigate the influence of glycolipid metabolism on function and expression of MDR1 and P-glycoprotein. The present study shows that MDR1 and

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P-glycoprotein expression can be down-regulated by GCS antisense transfection or chemical inhibition of GCS. We suggest that this avenue contributes to antisense GCS chemosensitization of drug-resistant cells. Overall, our work shows that lipids play a role in multidrug resistance and that targeting glycolipid biosynthesis could be a promising approach for enhancing chemotherapy.

Materials and Methods

Cell cultures. The MCF-7-AdrR human breast adenocarcinoma cell line, which is resistant to doxorubicin (20), was kindly provided by Dr. Kenneth Cowan (UNMC Eppley Cancer Center, Omaha, NE) and Dr. Merrill Goldsmith (NIH, Bethesda, MD). MCF-7-AdrR cells were maintained in RPMI 1640 (Invitrogen, Chicago, IL) containing 10% (v/v) fetal bovine serum (FBS; HyClone, Logan, UT), 100 units/mL penicillin, 100 μg/mL streptomycin, and 584 mg/L L-glutamine. Cells were cultured in a humidified, 5% CO_2 atmosphere tissue culture incubator and subcultured weekly using trypsin-EDTA (0.05%, 0.53 mmol/L) solution. MCF-7-AdrR cells transfected with GCS antisense (MCF-7-AdrR/GCS antisense) were cultured in RPMI 1640 containing the above components and G418 (400 μg/mL; ref. 16).

The KB-V0.01 multidrug-resistant human epidermoid carcinoma cell line (21) was grown in high glucose (4.5 g/L) DMEM with 10% fetal bovine serum and vinblastine (10 ng/mL). Cells were a gift from Dr. Michael Gottesman (National Cancer Institute, Bethesda, MD).

Cytotoxicity assays. Assays were done as described previously (13). Briefly, cells were seeded in 96-well plates (5,000 cells per well) in 0.1 mL RPMI 1640 containing 10% FBS and cultured at 37°C for 24 hours before addition of vinblastine sulfate or paclitaxel (Taxol), both of which were obtained from Sigma (St. Louis, MO). Drugs were added in FBS-free medium (0.1 mL), and cells were grown at 37°C for the indicated periods. Drug cytotoxicity was determined by the CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Madison, WI). Absorbance at 490 nm was measured by a fluorescent reader (Microplate FL600, Bio-Tek, Winooski, VT).

Analysis of lipids. Analysis was done as described previously (13, 22). Briefly, for ceramide, sphingomyelin, phospholipid, and neutral lipid analysis, cells were seeded 6-well plates (60,000 cells per well) in 10% FBS RPMI 1640. After 24 hours, medium was replaced with 5% FBS medium containing [^{3}H]palmitic acid (1.0 μCi/mL culture medium; DuPont/NEN, Boston, MA) for 24 hours. Total lipids were extracted as described (22), and the resulting organic lower phase was withdrawn and evaporated under a stream of nitrogen. Lipids were resuspended in 0.1 mL chloroform/methanol (2:1, v/v), and resolution was by TLC using solvent systems described (23). Commercial lipid standards (Avanti Polar Lipids, Alabaster, AL) were cochromatographed. After development, lipids were visualized by iodine vapor staining, and the area of interest was scraped into 0.5 mL water. EcoLume scintillation fluid (4.5 mL; ICN, Costa Mesa, CA) was added, samples were mixed, and radioactivity was quantitated by liquid scintillation spectrometry. For ceramide quantitation, cells were grown in 15-cm dishes, and total lipids were extracted and analyzed by TLC using a solvent system containing chloroform/acetic acid (90:10, v/v). Approximately 880 μg of lipid were loaded per lane. After development, TLC plates were sprayed with conc. sulfuric acid, and heated in an oven (30 minutes, 180°C). The ceramide chars were quantitated by scanning densitometry, using a ceramide standard curve (1.0-6.0 μg). Silica Gel G prescored TLC plates were purchased from Analtech (Newark, DE). Before the above procedure, plates were acid washed by running in methanol/HCl (90:10, v/v), and thoroughly dried before use.

Western blot for P-glycoprotein. Confluent monolayers of MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells were rinsed, harvested in PBS, and lysed in a PBS buffer containing 10% glycerol, 1% Triton X-100, 1.0 mmol/L Na_3VO_4, 10 mmol/L β-glycerophosphate, 50 mmol/L NaF, 0.1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, and 10 μg/mL aprotinin for 30 minutes on ice. The mixture was centrifuged at 11,000 × *g* for 15 minutes at 4°C. Equal aliquots of protein (25 μg) were resolved using 4% to 20% gradient SDS-PAGE (Invitrogen, Chicago, IL). The transferred nitrocellulose blot was blocked with 5% fat-free milk powder in PBS containing 0.1% Tween 20, at room temperature for 1 hour. The membrane was immunoblotted with 0.7 μg/mL of C219 murine monoclonal antibody against human P-glycoprotein (Calbiochem, Pasadena, CA) in the same blocking solution. Detection was done using enhanced chemoluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

RNA analysis. Total RNA was isolated using the RNeasy Protect Mini Kit from Qiagen, Inc. (Los Angeles, CA). MDR1 reverse transcription-PCR (RT-PCR) was carried out by a one-step method (SuperScript One-Step RT-PCR with Platinum Taq; Invitrogen, Chicago, IL). Total RNA (25 ng) was added to buffer containing 0.2 mM deoxynucleotide triphosphate, 1.2 mmol/L $MgSO_4$, 1.0 μL SuperScript II RT/Platinum Taq mix (containing reverse transcriptase and platinum Taq DNA polymerase), 0.2 μg of MDR1 upstream primer 5′-CCATCATTGCAATAGCAGG-3′, and 0.2 μg of MDR1 downstream primer 5′-GAGCATACATATGTTCAAACTTC-3′. RT-PCR, in a total volume of 50 μL, was done for 35 cycles in a thermocycler (Eppendorf Scientific, Westbury, NY); each cycle comprised denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minute. RT-PCR products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide. *β-Actin* primer (Stratagene, Cedar Creek, TX) was used as a housekeeping gene.

Real-time PCR. A real-time quantitative PCR analysis was done using the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Primers and probe sequences for MDR1 were chosen as follows: MDR1 forward 5′-GGTTTATAGTAGGATTTACACGTGGTTG-3′, MDR1 reverse 5′-AAGATAG-TATCTTTGCCCAGACAGC-3′, and MDR1 probe 5′ FAM CTAACCCTTGT-GATTTTGGCCATCAGTCC Tamra 3′. Human β-actin was used as endogenous control. Both assays used the SuperScript III Platinium one-step quantitative RT-PCR system (Invitrogen, Chicago, IL).

Glucosylceramide synthase gene silencing by small interfering RNA. The small interfering (siRNA) sequence targeting human GCS was selected using the BLOCK-IT RNAi Designer program; reagents were synthesized by Invitrogen (Carlsbad, CA). The siRNA duplex with the following sense and antisense sequences was used: 5′-CCAGGAUAUGAAGUUGCAA (sense) and 5′-UUGCAACUUCAUAUCCUGG (antisense). Established protocols were followed (24, 25). Briefly, siRNA was introduced into cells using LipofectAMINE 2000 in serum-free medium for 4 hours. FBS was added, and after 48 hours, total RNA was extracted and used to examine GCS and MDR1 mRNA levels. LipofectAMINE 2000 alone and expression of *β-actin* were used as controls.

Purification and analysis of gangliosides. Cells harvested in PBS were homogenized in 6 mL chloroform/methanol (1:1, v/v); the mixture remained overnight at room temperature. After centrifugation, the supernatant was dried and the lipid residue was taken up in chloroform/methanol (1:1) and centrifuged to remove all solid particles. Addition of PBS in a volume ratio of 1:1:0.7 (chloroform/methanol/PBS) separated the organic phase from the ganglioside-containing aqueous phase, as previously described (26). After thorough vortex mixing, the tube was centrifuged, and the upper phase containing gangliosides was withdrawn. Partitioning was repeated twice, each time by adding methanol/PBS (1:0.7, v/v) to the lower phase, followed by centrifugation. The upper phases were pooled, and gangliosides were recovered from the aqueous solution by column chromatography on C18-bonded silica gel. Ganglioside profiles were determined by high-performance TLC on Silica Gel 60 plates (Merck, Darmstadt, Germany) developed in chloroform/methanol/0.2% aqueous calcium chloride (55:45:10, v/v/v) and sprayed with resorcinol-HCl reagent. Ganglioside sialic acid content was determined by the periodate-resorcinol method (27).

[^{3}H]Paclitaxel and [^{3}H]vinblastine uptake studies. Cells were seeded into 12-well plates at 100,000 cells per well in 1.0 mL of complete medium. After 24 hours at 37°C, the medium was removed; cells were rinsed with PBS and incubated for 10 to 90 minutes with 0.5 mL of 5% FBS RPMI 1640 containing 500 nmol/L paclitaxel plus 0.25 μCi [^{3}H]paclitaxel (Moravek Biochemical, Brea, CA), or 500 nmol/L vinblastine plus 0.25 μCi [^{3}H]vinblastine (Moravek biochemical). After removal of culture medium, cells were washed twice with 5% FBS RPMI 1640 and lysed with 0.2 mL of 5% FBS RPMI 1640 containing 0.02% SDS. Intracellular radioactivity was measured by liquid scintillation counting.

MDR1 induction by glycolipids. KB-V0.01 cells were seeded into 6.0-cm dishes in complete medium. After 24 hours, medium was removed and cells were incubated in 5% FBS DMEM medium containing either 30 μg/mL C8 β-D-glucosylceramide (Avanti Polar Lipids, Alabaster, AL), 0.5 μmol/L doxorubicin (LKT Laboratories, St Paul, MN) as positive control, or 10 μg/mL palmitic acid (Sigma) as negative control. Cells were treated for 48 hours, and RNA was analyzed by real-time RT-PCR.

Chemical inhibition of glucosylceramide synthase. D,L-*Threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) was from Biomol Research Laboratories (Plymouth Meeting, PA) and D,L-*erythro*-PPMP and D-*threo*-PPMP were from Matreya (Pleasant Gap, PA). These reagents were used as described in the figure legends.

Statistical analyses. Student's *t* test was used for statistical analysis.

Results

Previously, we showed that GCS antisense transfection of multidrug-resistant MCF-7-AdrR cells enhanced cell sensitivity to doxorubicin, vinblastine, and paclitaxel (19). A doxorubicin-induced increase in ceramide levels and caspase activity is in keeping with ceramide-mediated cytotoxic responses to chemotherapy (16), but it is not clear whether ceramide is the only factor involved in the significantly (>100-fold) increased sensitivity of GCS antisense–transfected cells to *Vinca* alkaloids and paclitaxel.

Initial studies on ceramide production measured with [^{3}H]palmitate showed that similar levels of tritiated ceramide were formed in both MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells in response to vinblastine and paclitaxel challenge (Fig. 1*A*). Using mass analysis, however, we found that ceramide production in response to paclitaxel (1.0 μmol/L) was enhanced 3-fold in MCF-7-AdrR/GCS antisense cells compared with parental MCF-7-AdrR cells after 24 hours of treatment (Fig. 1*B*). To further assess the influence of GCS antisense transfection on cell response to chemotherapy, we next measured uptake and efflux variables of vinblastine and paclitaxel. P-glycoprotein–mediated drug efflux is the most widely characterized drug resistance mechanism in cancer cells (28), and it is highly expressed in MCF-7-AdrR cells (13). We previously reported that levels of rhodamine-123, a substrate of P-glycoprotein, were ~5-fold higher in MCF-7-AdrR/GCS antisense compared with MCF-7-AdrR cells (19). This suggests that GCS antisense transfection alters drug uptake and/or retention. Experiments with chemotherapy drugs showed that after 60 minutes, intracellular levels of vinblastine were 12-fold greater in MCF-7-AdrR/GCS antisense compared with MCF-7-AdrR cells (Fig. 2). Similarly, uptake of paclitaxel increased 8.6-fold in GCS antisense transfectants compared with MCF-7-AdrR cells (Fig. 2).

Because of the pronounced differences in drug levels in the two cell lines, we assessed P-glycoprotein expression, by mRNA and protein. As shown in Fig. 3, the level of MDR1 mRNA, evaluated by reverse transcription-PCR (RT-PCR), was dramatically lower in MCF-7-AdrR/GCS antisense compared with MCF-7-AdrR cells. We confirmed this by Western blot; whereas MCF-7-AdrR cells contained characteristically elevated levels of P-glycoprotein, MCF-7-AdrR/GCS antisense cells were nearly devoid of P-glycoprotein. These data suggest that high drug levels attainable in MCF-7-AdrR/GCS antisense cells are a consequence of the dramatic decrease (~80%) in P-glycoprotein expression. Thus, the stable 30% decrease in GCS activity of MCF-7-AdrR/GCS antisense cells (16) seems to have an important influence on intracellular drug levels and on P-glycoprotein expression. For this reason, we investigated whether partial inhibition of GCS would cause other alterations, in particular, in membrane lipid composition.

Steady-state [^{3}H]palmitic acid radiolabeling (24 hours) of MCF-7-AdrR/GCS antisense and MCF-7-AdrR cells showed, in the former, a 30% decrease in sphingomyelin levels and a 44% decrease in the level of phosphatidylinositols (data not shown).

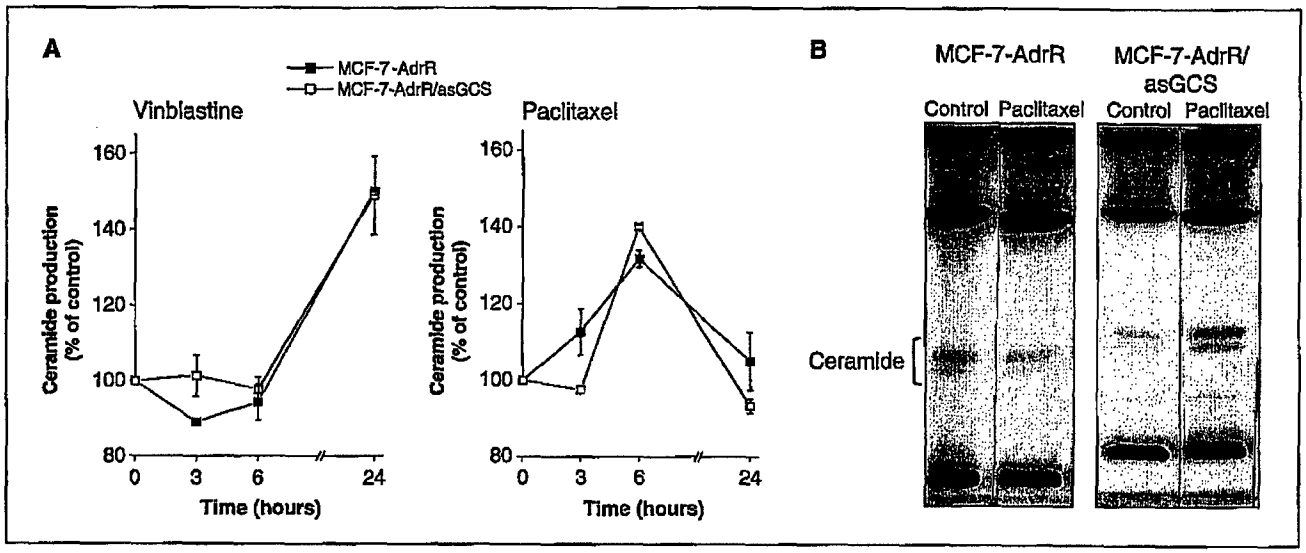

Figure 1. Influence of chemotherapy on ceramide formation in MCF-7-AdrR and MCF-7-AdrR/GCS antisense (*asGCS*) cells. *A*, assays using radiolabeling. Cells were treated with vinblastine (0.5 μmol/L) or paclitaxel (1.0 μmol/L) for the times shown in medium containing [^{3}H]palmitic acid. Tritiated ceramide was evaluated in the lipid extract by TLC and liquid scintillation spectroscopy as detailed in Materials and Methods. For comparison purposes, ceramide counts in MCF-7-AdrR control (no drug) were 11,890 ± 3,732 cpm/500,000 cpm total lipid and 11,084 ± 2,948 cpm/500,000 cpm total lipid in control (no drug) MCF-7-AdrR/GCS antisense cells. *Points*, mean from three experiments; *bars*, ±SD. *B*, ceramide assay using mass analysis. Cells were treated ± paclitaxel (1.0 μmol/L) for 24 hours and ceramide was evaluated by TLC sulfuric acid charring and photodensitometry as detailed in Materials and Methods. Ceramide mass in both MCF-7-AdrR control and MCF-7-AdrR paclitaxel-treated cells was 1.6 μg. Ceramide mass in MCF-7-AdrR/GCS antisense control and MCF-7-AdrR/GCS antisense paclitaxel treated was 1.0 and 3.25 μg, respectively. Total lipid (880 μg) was loaded in each lane.

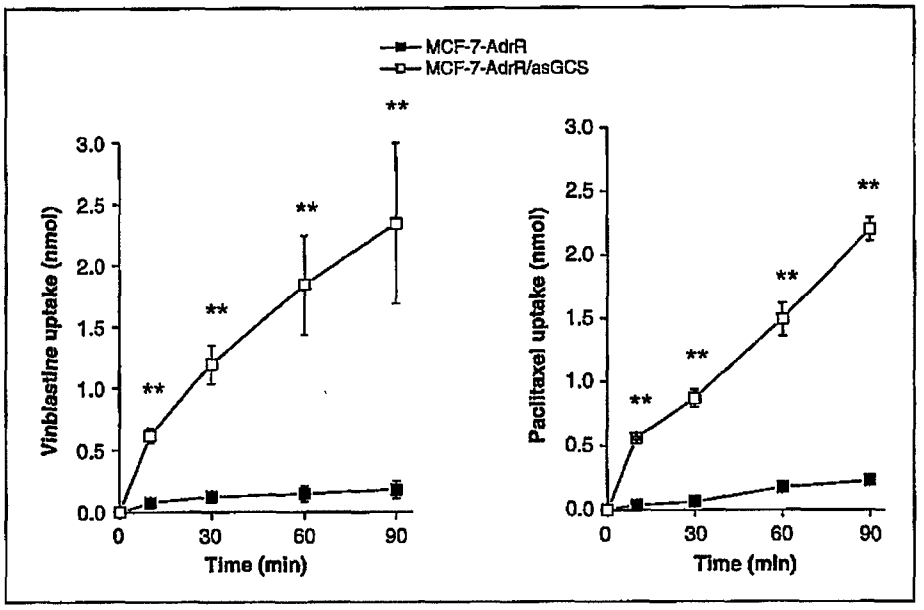

Figure 2. Uptake of vinblastine and paclitaxel in MCF-7-AdrR and MCF-AdrR/GCS antisense (*asGCS*) cells. *Points*, mean from two independent experiments; *bars*, ±SD. **, $P < 0.01$.

There were no significant differences in cholesterol esters or other glycerophospholipids between transfected and parent cells. Because GCS is pivotal in the genesis of cerebrosides and gangliosides, we also looked for changes in glycosphingolipid content. Although MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells expressed a similar ganglioside pattern (GM3, GM2, GD3, and GD1a; data not shown), sialic acid assay showed that the level of gangliosides was 4-fold lower in GCS antisense transfected cells (data not shown).

To determine whether depletion of gangliosides and reduced expression of P-glycoprotein were strictly a consequence of GCS down-regulation by antisense transfection, we evaluated the influence of D,L-*threo*-PPMP, a chemical inhibitor of GCS (29–31), on ganglioside synthesis and P-glycoprotein expression in MCF-7-AdrR cells. A 4-day exposure to D,L-*threo*-PPMP produced a 34% decrease in ganglioside levels in MCF-7-AdrR cells (Fig. 4*A*). Moreover, PPMP greatly diminished the expression of MDR1 in MCF-7-AdrR cells, with shown stereospecificity (Fig. 4*B*). Unlike D,L-*threo*-PPMP, D,L-*erythro*-PPMP is not a GCS inhibitor (32), and this stereoisomer had no influence on MDR1 expression. Real-time RT-PCR showed that MDR1 expression in MCF-7-AdrR cells treated with D,L-*threo*-PPMP and D,L-*erythro*-PPMP was reduced by 58% and 12%, respectively, compared with untreated MCF-7-AdrR cells (Fig. 4*B*). To reinforce the results obtained with PPMP and to confirm that changes in MCF-7-AdrR/GCS antisense cellular MDR1 expression were not due to clonal artifacts, we used GCS siRNA to treat MCF-7-AdrR cells. As shown in Fig. 4*C*, after 48 hours, both GCS and MDR1 mRNA were dramatically decreased by GCS siRNA compared with LipofectAMINE only controls. The siRNA had no effect on expression levels of β-actin.

Whether chemical lowering of MDR1 expression affects cellular response to chemotherapy was next evaluated. Treatment of MCF-7-AdrR cells for 4 days with D,L-*threo*-PPMP enhanced vinblastine uptake by ~3-fold at 30 and 60 minutes (Fig. 4*D*), and as illustrated in Fig. 4*E*, vinblastine cytotoxicity, even at low concentrations (0.1 μmol/L), was enhanced ~60% in cells that had been cultured with D,L-*threo*-PPMP. Thus, like GCS antisense transfection, inhibition of GCS by chemical means reversed resistance of MCF-7-AdrR cells to vinblastine. To examine the generality of this response, we used KB-V0.01 cells, a head/neck multidrug-resistant epidermoid carcinoma cell line that expresses both GCS and MDR1 (33). As shown in Fig. 5*A*, MDR1 expression in KB-V0.01 cells was lowered 70% by D-*threo*-PPMP (10 μmol/L) and 38% by D,L-*threo*-PPMP (15 μmol/L). Therefore, the D-*threo* isomer is the most effective inhibitor of GCS compared with the racemic mixture. KB-V0.01 cell treatment with D-*threo*-PPMP for a prolonged period (7 days) induced a dramatic decrease (84%) in MDR1 mRNA levels (Fig. 5*B*). Moreover, P-glycoprotein protein levels in these cells diminished by 50%, compared with the untreated control (Fig. 5*C*). We next investigated the effect of glycolipid supplementation on MDR1 expression. Growth of KB-V0.01 cells with cell-permeable C8-glucosylceramide (30 μg/mL) elicited a 2-fold increase in MDR1 mRNA levels (Fig. 6), a response nearly comparable to the influence of Adriamycin (0.5 μmol/L) on MDR1 expression (Fig. 6). Palmitic acid, used as a lipid control, had no influence on MDR1 expression.

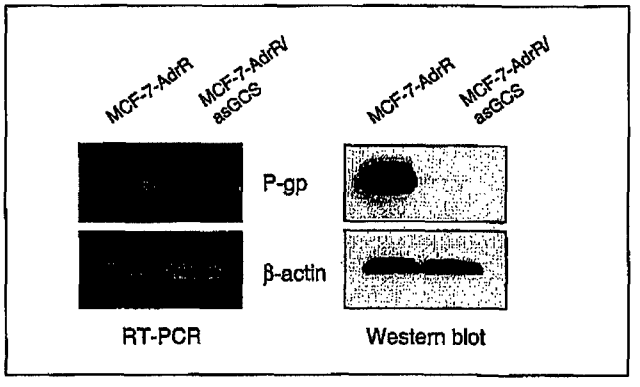

Figure 3. P-glycoprotein (*P-gp*) expression in MCF-7-AdrR and MCF-7-AdrR/GCS antisense (*asGCS*) cells. MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells were grown to 70% confluence, and RNA and protein were extracted and used for RT-PCR and Western blot analysis. The C219 murine monoclonal antibody against human P-glycoprotein was used for the Western blot.

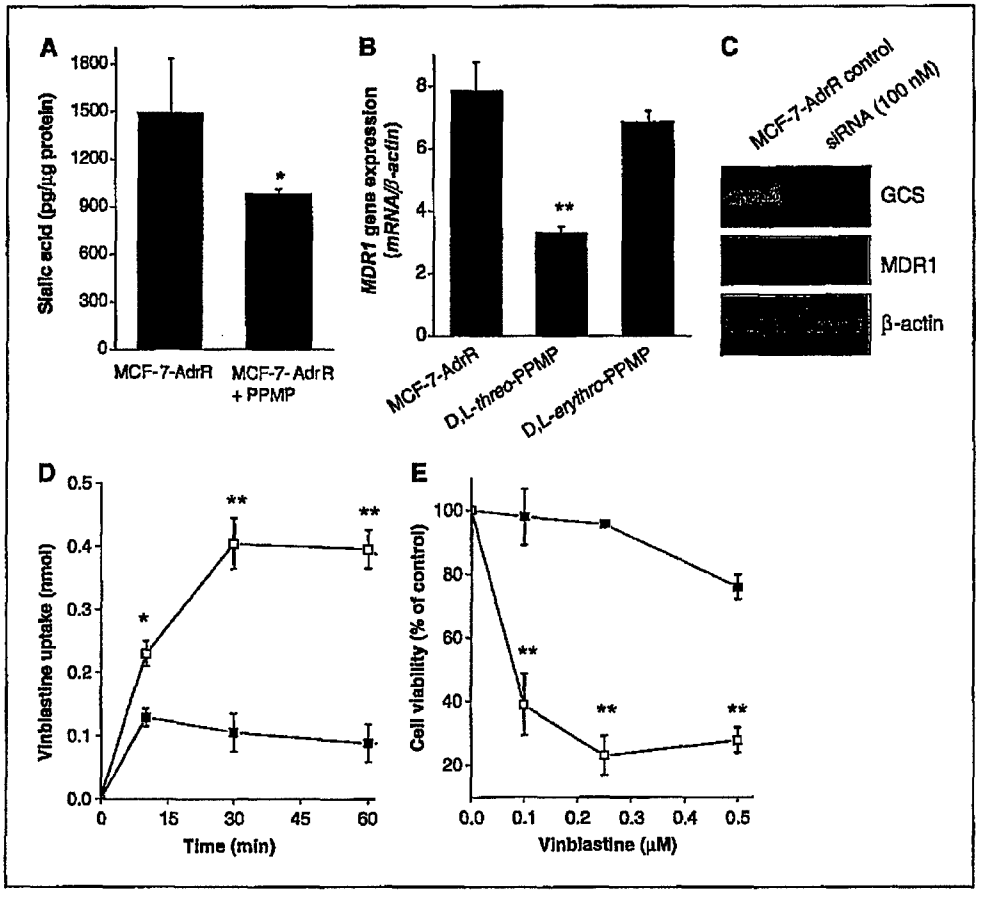

Figure 4. Influence of GCS blockade via PPMP and siRNA on ganglioside levels, MDR1 expression, and drug uptake and chemosensitivity in multidrug-resistant MCF-7-AdrR cells. *A* and *B*, influence of PPMP on ganglioside levels and MDR1 expression. *A*, MCF-7-AdrR cells were incubated with D,L-*threo*-PPMP (5.0 μmol/L, 96 hours). Gangliosides were extracted and quantified by sialic acid derivitization. PPMP treatment did not alter cell doubling time. *B*, MDR1 mRNA levels in MCF-7-AdrR cells incubated in the absence and presence of either D,L-*threo*-PPMP or D,L-*erythro*-PPMP (15 μmol/L, 48 hours). D,L-*erythro*-PPMP did not influence cell growth; the *threo* form retarded cell growth 20%, compared with control. RNA was extracted and quantified by real-time RT-PCR. *C*, influence of GCS siRNA (100 nmol/L, 48 hours) on GCS and MDR1 mRNA expression in MCF-7-AdrR cells. LipofectAMINE alone was used as control. *D*, vinblastine uptake was determined in MCF-7-AdrR cells preincubated with D,L-*threo*-PPMP (5.0 μmol/L, 96 hours). PPMP did not alter cell growth. *E*, vinblastine cytotoxicity in MCF-7-AdrR cells preincubated with D,L-*threo*-PPMP (5.0 μmol/L, 96 hours) and exposed to vinblastine at the indicated concentrations for 3 days. Cell viability was determined by MTS assay. *Columns*, mean of triplicates from two independent experiments; *bars*, ±SD. *, $P < 0.05$; **, $P < 0.01$.

Discussion

The present study shows that GCS antisense transfection of multidrug-resistant human breast cancer cells modifies cellular lipid composition, reduces MDR1 expression, and enhances the cytotoxic effect of chemotherapeutic drugs. GCS antisense transfection decreased the levels of sphingomyelin in MCF-7-AdrR cells. Sphingomyelin is a major constituent of the external leaflet of the plasma membrane (34). Sphingomyelin, phosphatidylcholine, and proteins are laterally organized in biological membranes (35–37). These organized domains have been suggested to participate in cellular processes, such as signal transduction, membrane trafficking, and protein sorting (38). Expression of the principal component of caveolae, caveolin-1, in MCF-7-AdrR and MCF-7-AdrR/GCS antisense cells, determined by Western blot, was not affected by antisense down-regulation of GCS (data not shown), although it should be mentioned that this assay is not a good estimate of the status of cellular lipids in rafts/caveolae. However, we found that transfected cells had lower levels of gangliosides, the sialic acid–containing glycosphingolipids. Gangliosides have been shown to influence lipid order and hydration of the lipid bilayer; such changes could play an important role in modulation of transmembrane molecular events (39). Moreover, gangliosides have been shown to influence membrane fluidity (40–42). Cellular ganglioside levels decreased 4-fold in MCF-7-AdrR/GCS antisense cells compared with MCF-7-AdrR cells. Such a change could modify membrane permeability and facilitate entrance of natural-product chemotherapeutic agents such as vinblastine and paclitaxel.

In addition to their role as a structural component of the plasma membrane, gangliosides might regulate signaling events. In melanoma cells, transient ganglioside depletion by GCS inhibition reduced tumorigenic capacity (43). Gangliosides can also induce production of nitric oxide, tumor necrosis factor-α, and cyclooxygenase 2 and activate extracellular signal–regulated kinase and c-*jun*/stress-activated protein kinase kinase, p38, and nuclear factor κB (NF-κB; ref. 44). Our work showed that inhibiting the activity of GCS severely limited the expression of MDR1 and its product, P-glycoprotein. Studies have shown that the MDR1 promoter can be activated directly by anticancer agents such as vincristine, daunorubicin, doxorubicin, and colchicine (45);

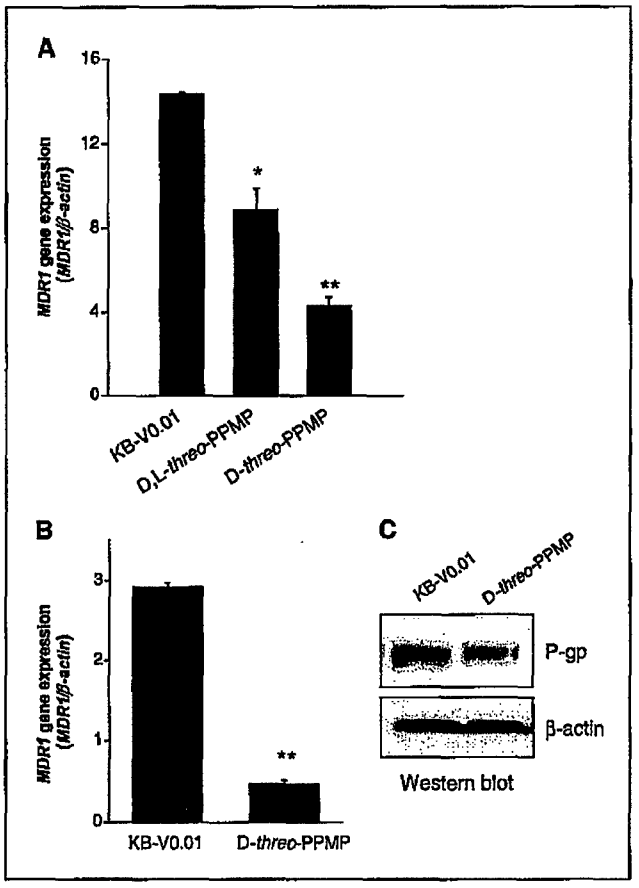

Figure 5. Influence of PPMP on MDR1 mRNA and protein (P-glycoprotein) levels in KB-V0.01 cells. *A*, MDR1 mRNA levels in KB-V0.01 cells incubated in the absence and presence of either D-*threo* PPMP (10 μmol/L, 72 hours) or D,L-*threo* PPMP (15 μmol/L, 72 hours). Measurements were made by real-time RT-PCR. Compared with control, D-*threo* PPMP and D,L-*threo* PPMP reduced cell growth by 20% and 40%, respectively. *B* and *C*, KB-V0.01 cells were grown with D-*threo* PPMP (10 μmol/L) for 7 days, and RNA and protein were extracted and used for real-time RT-PCR (*B*) and Western blot analysis (*C*). Seven-day exposure to D-*threo* PPMP, which necessitated a medium change, increased cell doubling time by twice, compared with controls. *Points*, mean from two independent experiments; *bars*, ±SD. *, $P < 0.05$; **, $P < 0.01$.

however, an association between glycolipids and the MDR1 promoter has not been clearly established. Some studies have suggested that glycolipids, in particular gangliosides, modulate multidrug resistance. For example, the up-regulation of GM3 biosynthesis in fenretinide-adapted A2780 ovarian cancer cells has been correlated with fenretinide resistance (46). In human leukemia cells, ganglioside depletion is believed to account for PDMP-mediated reversal of multidrug resistance, and GM3 and GD3 are thought directly involved via modulation of P-glycoprotein function through phosphorylation (47). Shabbits et al. (48) showed a relationship between drug transport and ceramide metabolism. Other support for a link between glycolipids and multidrug resistance may be found in the PPMP-modulated expression of MDR1 mRNA in SKOV3/AdrR human ovarian cancer cells (49), in KBV200 cells (50), and in the decreased efflux of [^{14}C]paclitaxel and [^{3}H]vincristine in a neuroblastoma cell model (51). Results of other studies show that verapamil, an antihypertensive formerly used clinically as a P-glycoprotein antagonist (52), limits the expression of MDR1 in human leukemia cells (53). Our group showed that verapamil, tamoxifen, and cyclosporine A block glucosylceramide formation and resultant downstream cerebroside and ganglioside biosynthesis in drug-resistant cancer cells (54).

Previously, we showed that doxorubicin treatment of MCF-7-AdrR/GCS antisense cells enhanced the production of ceramide (16). In the present study using radiolabeling, ceramide buildup was not evident in MCF-7-AdrR/GCS antisense cells challenged with either vinblastine or paclitaxel (Fig. 1*A*); however, lipid mass analysis by TLC char clearly showed elevated ceramide levels in drug-challenged GCS antisense transfectants (Fig. 1*B*). Failure of radiolabeling techniques to accurately portray mass is not uncommon. More importantly however is the apparent dual role that GCS antisense transfection and/or GCS blockade play in sensitizing multidrug-resistant cancer cells to chemotherapy. From our experiments, it is evident that GCS antisense (i) down-regulates expression of MDR1 and (ii) promotes ceramide buildup in cells that would otherwise scavenge ceramide via elevated GCS activity. This one-two punch could be of benefit in cancer treatment.

The present results suggest that glycolipids participate in MDR1 expression directly or via activation of a specific transcription factor. In a recent study, Bentires-Alj et al. (55) showed that NF-κB inhibition increased cellular uptake of daunorubicin and reduced expression of MDR1 mRNA and protein (P-glycoprotein) in colon cancer cells. NF-κB complexes can bind at a consensus NF-κB binding site in the first intron of the human *MDR1* gene. Moreover, NF-κB can transactivate an MDR1 promoter luciferase construct (55).

In conclusion, our work shows that limiting GCS activity by either GCS antisense transfection, siRNA transfection, or PPMP treatment down-regulates the expression of P-glycoprotein. It should be noted, however, that drug resistance through enforced overexpression of GCS in wild-type MCF-7 cells, using a retroviral tetracycline-on expression system, did not rely on P-glycoprotein induction (13) but rather on ceramide scavenging. In addition, GCS antisense transfection retards clearance of ceramide generated in response to chemotherapeutics such as paclitaxel. Therefore, ceramide-signaled death cascades and depletion of cellular P-glycoprotein are likely contributory to heightened chemosensitivity in MCF-7-AdrR/GCS antisense. We propose that overexpression of GCS contributes to chemotherapy resistance by

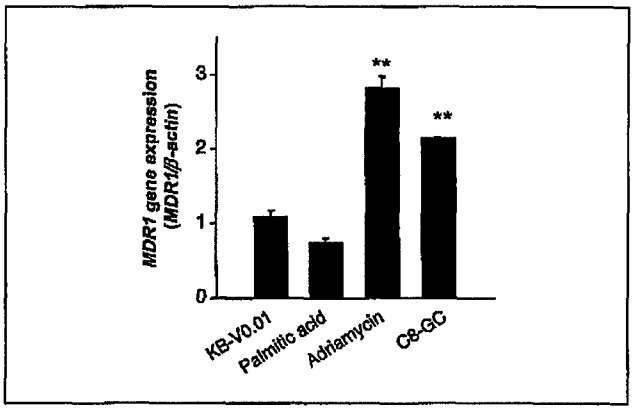

Figure 6. Influence of C8-glucosylceramide supplement on MDR1 expression in KB-V0.01 cells. Cells were incubated for 48 hours with either C8-GC (30 μg/mL), Adriamycin (0.5 μmol/L) used as positive control, or palmitic acid (10 μg/mL) used as lipid control. RNA was extracted and analyzed by real-time RT-PCR. C8-GC was not cytotoxic at the employed dose. *Columns*, mean from two independent experiments; *bars*, ±SD. **, $P < 0.01$.

enhancing levels of cerebrosides and/or gangliosides that could promote the expression of MDR1. Doxorubicin-activated expression of GCS in MCF-7 drug-sensitive cells (56) lends support to this novel slant on the metabolism of ceramide.

Acknowledgments

Received 6/30/2004; revised 2/8/2005; accepted 2/24/2005.

Grant support: USPHS/National Cancer Institute grant CA95339, Department of Defense/U.S. Army postdoctoral award (V. Gouazé), the Susan G. Komen Breast Cancer Foundation, Associates for Breast and Prostate Cancer Studies (Santa Monica, CA), Sue and Larry Hochberg, and Fashion Footwear Association of New York Charitable Foundation (NY).

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We thank Dr. Jacques Portoukalian (Institut National de la Sante et de la Recherche Medicale U346, Lyon, France) for helpful discussions.

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Glucosylceramide synthase blockade downregulates P-glycoprotein and resensitizes multidrug-resistant breast cancer cells to anticancer drugs

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Overexpression of glucosylceramide synthase (GCS), a pivotal enzyme in glycolipid biosynthesis, contributes to cancer cell resistance to chemotherapy. We previously demonstrated that transfection of doxorubicin-resistant MCF-7-AdrR cells with GCS antisense (asGCS) restored cell sensitivity to doxorubicin and greatly enhanced sensitivity to vinblastine and paclitaxel. In that study, doxorubicin promoted generation of ceramide in MCF-7-AdrR/asGCS cells; the present study implicates factors other than ceramide in the sensitivity of asGCS-transfected cells to chemotherapy. Although asGCS cells demonstrated enhanced ceramide formation compared to MCF-7-AdrR when challenged with paclitaxel, asGCS cells also demonstrated a 10-fold increase in levels of intracellular drug (paclitaxel, vinblastine). In addition, transfected cells had dramatically decreased (80%) expression of P-glycoprotein (P-gp), and a 4-fold decrease in the level of cellular gangliosides. Chemical inhibition of GCS produced the same effects as antisense transfection: exposure of MCF-7-AdrR cells to the GCS inhibitor PPMP (1-phenyl-2-palmitoylamino-3-morpholino-1-propanol) (5.0 μM; 4 days) decreased ganglioside levels, restored sensitivity to vinblastine, enhanced vinblastine uptake 3-fold, and diminished expression of MDR1 by 58%, compared to untreated controls. A similar effect was demonstrated in vinblastine-resistant KB-V0.01 cells; after 7 days with PPMP (10 μM), MDR1 expression fell by 84% and P-gp protein levels decreased by 50%. MCF-7-AdrR cells treated with small interfering RNAs to specifically block GCS also showed a dramatic decrease in MDR1 expression. This is the first evidence that limiting GCS activity downregulates the expression of MDR1, a mechanism that may drive the chemosensitization associated with targeting ceramide metabolism. The data suggest that lipids play a role in multidrug resistance, and that limiting ganglioside biosynthesis could be a promising approach for reversing multidrug resistance in cancer.